

# Avoidance of false PCR results with the integron–retron junction in multiple antibiotic resistant *Salmonella enterica* serotype Typhimurium<sup>☆</sup>

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## Abstract

*Salmonella* infections continue to cause gastrointestinal and systemic disease throughout the world. Another concern with this pathogen is the ability to acquire integrons that confer resistance to multiple antibiotics. For multiresistant *Salmonella enterica* serotype Typhimurium, the most common multiresistant *Salmonella* serotype, an integron structure can be found between *thdF* and a retron. Our objective was to investigate the utility of a 450 bp *thdF*-retron amplicon as an indicator of an insertless *thdF*-retron junction thus indicating an integron-free strain. Surprisingly, we found that the 450 bp *thdF*-retron amplicon was present, and thus incorrectly suggesting an integron-free status, in some multiresistant *S. enterica* serotype Typhimurium isolates. However, this phenomenon was not observed if the isolate was enriched in the presence of two antibiotics. This demonstrates that, within some individual clinical isolates of multiresistant *S. enterica* serotype Typhimurium, there exists a small subpopulation of integron-free bacteria. Consequently, it appears that the *thdF*-retron amplicon is an inaccurate predictor of integron status in *S. enterica* serotype Typhimurium unless multiresistance is used as a selection tool during enrichment.

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**Keywords:** Integron; Retron; *Salmonella*; Antibiotic resistance; PCR

## 1. Introduction

*Salmonella* infections continue to be a problem in both industrialized and developing countries [1]. This problem is exacerbated by the ability of *Salmonella* to become resistant to multiple antibiotics. The current paradigm for this problem is *Salmonella enterica* serotype Typhimurium DT104 (DT104), a pathogen with a wide range of hosts and a wide range of antibiotic resistances (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline; ACSSuT antibiogram).

For DT104, the ACSSuT antibiogram is determined by a genomic collection of adjacent genes [2–4]. This

arrangement is composed largely of two integrons containing the genes *pse-1*, *floR*, *aadA2*, *sul1*, and *tetA* that, respectively, encode resistance to ampicillin, chloramphenicol/florfenicol, streptomycin/spectinomycin, sulfonamides, and tetracycline. Insertion of the integrons into DT104 occurs between *thdF* and a retron in the genome [5]. The former is a gene involved in furan oxidation in *E. coli* [6] and the latter is a Typhimurium-specific segment of DNA [5] introduced as a result of an infection with an RNA bacteriophage [7]. Thus the *thdF*-retron junction may serve as a preferred site for DNA insertion.

Our original intent was to exploit the *thdF*-retron interface in order to ascertain the integron status of isolates of *S. enterica* serotype Typhimurium. Instead, we identified the existence of an integron-free subpopulation within individual isolates of DT104. This subpopulation was not evident when the isolate was enriched in the presence of two antibiotics.

<sup>☆</sup> Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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## 2. Materials and methods

**Bacterial strains.** Multiresistant strains of *S. enterica* serotype Typhimurium (phage types DT104, U302, DT120, DT193 and DT208) were obtained from the National Veterinary Services Laboratories (Ames, IA). Multiresistance was defined as resistance to at least five antibiotics. Strains were obtained from clinical and environmental sources in 1997, 1998 and 2001. A synopsis of the 1997–1998 strain collection can be found in Frana et al. [8]. A similar collection was obtained in the year 2001.

**DNA isolation.** Purified DNA was isolated from Lennox L broth (GIBCO-BRL) broth using the GNome kit (Bio101, Vista, CA) as per manufacturer's protocol. DNA concentration was determined using the spectrophotometric analysis of Kalb and Bernlohr [9].

**PCR.** Sequences and locations of oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) are described in Table 1. PCR was performed in an automated thermocycler (Hybaid, Teddington, UK) with a hot bonnet. PCR reactions were performed in 0.2 ml tubes with 20 µl containing 300 µM dATP, dTTP, dCTP and dGTP, 2.5 mM magnesium chloride, 4 pmoles of each primer, 10 mM Tris–HCl, 50 mM KCl, 0.5 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA) and 50 ng of template (or water as a negative control). Thermocycling entailed 95 °C for 5 min, then 40 cycles of 95 °C for 1 min, 48 °C for 30 s and 72 °C for 30 s.

**Agarose gel electrophoresis.** PCR products (10 µl of the reaction) were electrophoresed in 2.0% agarose (Perkin Elmer) gels for 1.5 h at 150 V with Tris (40 mM)–acetate (20 mM)–EDTA (1 mM) as the running buffer. Ethidium bromide-stained amplicons were visualized on a UV transilluminator using the GelDoc system (BioRad, Richmond, CA).

**DNA sequence analysis.** The *thdF*-retron segment was amplified by PCR using the forward and reverse primers described in Table 1. PCR was performed as described previously [10]. PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) as per manufacturer's protocol. Plasmids containing the appropriate size inserts were sequenced by Iowa State University DNA Sequencing and Synthesis Facility using the vector-specific primers T7 and M13.

**Antibiotic resistance assays.** Minimum inhibitory concentration (MIC) values were determined by inoculating 10<sup>6</sup> bacteria into 1 ml aliquots of Mueller–Hinton broth

(DIFCO, Detroit, MI) containing two-fold serial dilutions of antibiotics as per the National Committee on Clinical Laboratory Standards guidelines [11]. Bacteria were grown aerobically and MIC values were based on the lowest concentration of antibiotic that inhibited growth. Resistance breakpoints, obtained from the National Committee on Clinical Laboratory Standards are: 32 µg/ml for ampicillin; 32 µg/ml for chloramphenicol; 64 µg/ml for streptomycin; 512 µg/ml for sulfamethoxazole; 16 µg/ml for tetracycline. All antibiotics were obtained from Sigma (St Louis, MO).

## 3. Results

**PCR for detecting the integron in *S. enterica* serotype Typhimurium.** During an assessment of the *thdF*-retron as an indicator of integron insertion in *S. enterica* serotype Typhimurium, we noticed that a strain of multiresistant DT104 exhibited an apparent amplicon in a PCR using primers spanning the *thdF*-retron junction. We assumed that the *thdF*-retron PCR product would be too large to be observed since this DT104 strain possessed the integron structure based on the presence of a *floR-tetR* amplicon [10]. Sequencing of the *thdF*-retron amplicon revealed the absence of an integron structure between *thdF* and the retron. As depicted in Fig. 1 and Table 2, several multiple antibiotic resistant isolates of DT104 also appear to have a subpopulation that lacks the integron. Specifically, these isolates have an amplicon from both the *thdF*-retron and *floR-tetR* PCRs. This genotype was found in 10 of 838 isolates from 1997 to 2001 and is represented by strain 418 in Fig. 1. Nine of the 10 isolates were obtained in 2001 while one isolate (strain 773) was obtained in 1997. All 10 isolates belong to the DT104 phage type, i.e. this phenomenon was not observed in the DT120, DT193, U302 phage types that are related to DT104 and possess the same integron structure as DT104.

As shown in the parentheses in Table 2, the *thdF*-retron/*floR-tetR* genotype was observed if these isolates were grown in the absence of antibiotics or in the presence of ampicillin, chloramphenicol, streptomycin, sulfamethoxazole or tetracycline (latter four not shown). However, only the *floR-tetR* genotype was observed if the isolate was grown in a combination of ampicillin and chloramphenicol. Similar results were obtained when the isolates were grown in a combination of streptomycin and tetracycline (data not shown). The inability to detect the *thdF*-retron amplicon

Table 1  
Nucleotide sequences of PCR primers used in this study

Primer pair	Forward	Reverse	Amplicon size	Reference
<i>thdF</i> -Retron	5'ACACCTTGAGCAGGGCAAAG	5'AGCAAGTGTGCGTAATTTGG	450 or 40 kb <sup>a</sup>	[5]
<i>floR-tetR</i>	5'CGCTCCTTCGATCCCGT	5'GCTGCGTTCATCTACAACAGAT	275 bp	[10]

<sup>a</sup> 450 bp indicates absence of the integron while 40 kb (i.e. no product) indicates the presence of the integron structure; no product also suggests the absence of the retron.

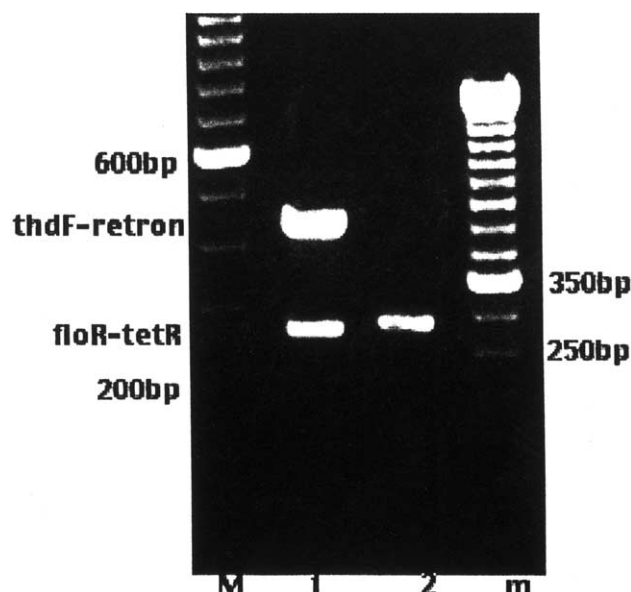


Fig. 1. Agarose gel electrophoresis of amplicons observed following multiplex PCR for genes in *S. enterica* serotype Typhimurium phage type DT104 strain 418. Lane 1 was derived from strain 418, enriched without antibiotics, while lane 2 was derived from strain 418 enriched in the presence of both ampicillin and chloramphenicol. Lanes designated as 'M' and 'm' represent 100 and 50 bp molecular weight standards (GIBCO BRL, Gaithersburg, MD), respectively. The locations of the *thdF*-retron and *floR*-*tetR* amplicons are indicated on the left. Strain 418, when grown only in ampicillin or only in chloramphenicol, yielded both amplicons in this multiplex PCR (not shown). Strains were obtained from sources described in Section 2.

was not related to alterations in *thdF* or the retron since both of these sequences were found to be intact when amplified with gene-specific primers (data not shown).

*Identification, characterization and quantification of individual colonies that lack the integron structure.* Individual isolates were plated on antibiotic-free media and then evaluated for colony morphology, ampicillin resistance, integron presence and phage type. One hundred colonies were examined for each of the 10 isolates displaying the *thdF*-retron/*floR*-*tetR* genotype. As shown in Table 2, 26 of 1000 (or 2.6%) of these colonies lacked the integron structure and thus were sensitive to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline. These colonies had a similar morphology to their cohorts and they all belong to the DT104 phage type like the rest of the population.

#### 4. Discussion and Conclusions

Since the *thdF*-retron junction appears to be a site for receiving large fragments of exogenous DNA in *S. enterica* serotype Typhimurium, we attempted to develop a PCR protocol that would reveal if an isolate possessed an insertion at this junction. Instead, we found that this strategy was not reliable due to integron inconsistencies in multi-resistant isolates from a collection of strains obtained in 1997–2001. This is in contrast to a recent study by Boyd

Table 2  
Status of integron presence in individual colonies of DT104 isolated in 1997–2001

Strain	Percentage of colonies sensitive to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline <sup>a</sup>	<i>thdF</i> -Retron	<i>floR</i> - <i>tetR</i>	Percentage of colonies resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline <sup>b</sup>	<i>thdF</i> -Retron	<i>floR</i> - <i>tetR</i>
418 <sup>c</sup>	5 (4, 0)	Positive	Negative	95 (96, 100)	Negative	Positive
3349 <sup>c</sup>	9 (5, 0)	Positive	Negative	91 (95, 100)	Negative	Positive
126 <sup>c</sup>	2 (2, 0)	Positive	Negative	98 (98, 100)	Negative	Positive
3356 <sup>c</sup>	3 (2, 0)	Positive	Negative	97 (8, 100)	Negative	Positive
4017 <sup>c</sup>	2 (1, 0)	Positive	Negative	98 (99, 100)	Negative	Positive
1006 <sup>c</sup>	1 (1, 0)	Positive	Negative	99 (99, 100)	Negative	Positive
1086 <sup>c</sup>	1 (1, 0)	Positive	Negative	99 (99, 100)	Negative	Positive
1221 <sup>c</sup>	1 (1, 0)	Positive	Negative	99 (99, 100)	Negative	Positive
2005 <sup>c</sup>	1 (1, 0)	Positive	Negative	99 (99, 100)	Negative	Positive
773 <sup>d</sup>	1 (1, 0)	Positive	Negative	99 (99, 100)	Negative	Positive
563 (control) <sup>d</sup>	0 (0, 0)	NA	NA	100 (100, 100)	Negative	Positive
<i>S. enterica</i> serotype Agona (multiresistant)	0 (0, 0)	NA	NA	100 (100, 100)	Negative <sup>e</sup>	Positive

<sup>a</sup> Numbers in parentheses represent percent of sensitive colonies after isolate enrichment in ampicillin alone or ampicillin plus chloramphenicol, respectively.

<sup>b</sup> Numbers in parentheses represent percent of resistant colonies after isolate enrichment in ampicillin alone or ampicillin plus chloramphenicol, respectively.

<sup>c</sup> From 2001.

<sup>d</sup> From 1997.

<sup>e</sup> Negative because this serotype does not possess the retron.

et al. [5] in which the *thdF*-retron amplicon was used as a marker for the integron structure.

Integron inconsistency was based on the presence of a *thdF*-retron amplicon in some isolates of multiresistant *S. enterica* serotype Typhimurium (especially phagetype DT104). The *thdF* gene lies on the 'left' side of the retron, a Typhimurium-specific gene segment [5] that is a result of a previous RNA bacteriophage infection [7]. For *S. enterica* serotype Typhimurium DT104, an integron structure is inserted between *thdF* and the retron [5]. Thus the presence of this structure would generate a 40-kb product which would not be observed using the PCR conditions employed.

In this study we identified two subpopulations within certain isolates. One subpopulation is positive for the *thdF*-retron amplicon and negative for the integron amplicon (*floR-tetR*) while the other subpopulation is negative for *thdF*-retron amplicon and positive for the integron amplicon. For the former subpopulation it appears that the integron structure is absent or inserted at an alternative site or that the retron has been translocated. It is possible for the integron structure to be inserted independent of the retron given the presence of the integron structure in multiresistant *S. enterica* serotype Agona [12], a strain that lacks the retron but possesses the integron structure adjacent to *thdF*. Retron translocation does not appear to be valid since the *thdF*-retron PCR indicates that the retron is adjacent to *thdF*. Retron-independent insertion of the integrons also appears invalid since the integron-specific PCR is negative and the colonies are antibiotic-sensitive. Thus it appears that some colonies do not possess the integron. Interestingly, this 'integron inconsistency' phenomenon was not observed in multiresistant *S. enterica* serotype Agona although only one isolate was examined.

The existence of the integron-free subpopulation can be eliminated by the use of multiple (e.g. ampicillin and chloramphenicol), but not single, antibiotics during the enrichment phase. Evidently the antibiotic-sensitive colonies are able to co-exist with antibiotic resistant colonies unless enrichment is performed with multiple selection pressures.

In summary, this study is the first to document an occasional inconsistency in the integron status in multi-resistant *S. enterica* serotype Typhimurium. The implications are two-fold. First, it now appears that it will be inappropriate to use the *thdF*-retron amplicon as a diagnostic tool unless two or more antibiotics are used during the enrichment phase. That is, the presence of this amplicon should not be used as a tool for examining *thdF*-retron insertion events in *S. enterica* serotype Typhimurium. Second, it appears that either the integrons are not stable or integron-free subpopulations co-exist with colonies possessing the integron structure. Thus these studies could

provide the basis for studying the genetic drift and population dynamics of multiresistant *S. enterica* serotype Typhimurium.

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